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MUTANT GENE OF THE GRAS FAMILY AND PLANTS WITH REDUCED DEVELOPMENT CONTAINING SAID MUTANT GENE

The invention relates to the production of plants with reduced development, and in particular of crucifers.

The use of dwarf plants in the context of agricultural production has many advantages; for example, in cereals, the use of short-straw mutant plants has made it possible to produce crops which tolerate considerable amounts of nitrogen-containing fertilizers, which are less affected by weather conditions and, in particular, more resistant to torrential rain than the plants which are normal in size. In addition, the small size of the plants facilitates the maintenance of the crops, in particular the application of plant-protection treatments, and also the harvesting thereof.

Dwarf mutants of plants other than cereals have also been described in the literature. Mention will in particular be made below of mutants which have characteristics similar to those induced by a deficiency in gibberellins and which are insensitive to the providing of exogenous gibberellins. Such mutants have in particular been described in *Arabidopsis* [Koornneef et al., Physiol. Plant., 65, 33-39, (1985)]. These mutants, named *gai* (for gibberellic acid insensitive) are smaller in size and do not respond to exogenous applications of gibberellins. The *gai* mutation is a semi-dominant mutation of the "gain of function" type. *GAI/gai* heterozygous mutants have an intermediate phenotype between that of *gai/gai* dwarf mutants and *GAI/gai* wild-type plants.

Mutants having the same characteristics as the *gai* mutants of *Arabidopsis* have been described by Zanewich et al. [J. Plant Growth Regul., 10, 121-127, (1991)],

in *Brassica napus* (*dwf1* mutation) and *Brassica rapa* (mutations named *dwf1* and *dwf2*).

The team of the inventors has obtained a dwarf mutant of *B. rapa* [Foisset et al., Theor. Appl. Genet., 91, 756-761, (1995)]. The mutation, named *bzh*, has characteristics of "semi-dominance" and of insensitivity to gibberellins, which are similar to those of the *gai* mutation.

A rapeseed line, named ISN1770, homozygous for the *bzh* mutant allele, has been the subject of a Certificat d'Obtention Végétale [Plant Variety Protection Certificate] filed on May 18, 1998, with the CPOV [French Plant Variety Protection Office] (11 rue Jean Nicaud, 75007 Paris) under the reference 10751. A rapeseed hybrid, named "Lutin" (B017), comprising in its genome the *bzh* mutant allele in heterozygous form was proposed for listing in the Catalogue Français des Obtentions Végétales [French Catalogue of Plant Varieties] on July 31, 1999, under the reference 072426.

The *GAI* gene of *Arabidopsis* has recently been cloned and sequenced [Peng et al., Genes and development, 11, 3194-3205 (1997); PCT application WO 97/29123 in the name of John Innes Centre Innovations Ltd.]. This gene encodes a 532 aa protein (GAI). The *gai* allele, which is responsible for dwarfism, contains a deletion of 51 base pairs in frame with the reading frame, which leads to the absence of 17 aas located close to the N-terminal end of the GAI protein. The GAI protein is involved in the perception of and response to gibberellins, and is thought to act, in wild-type plants, as a negative regulator of cellular elongation in the absence of gibberellins.

Comparison of the GAI sequence with that of the translation products of other known genes has made it

possible to place it in the family named GRAS [Pysh et al., The Plant Journal, 18(1), 11-119, (1999)] or VHIID [Schumacher et al., P.N.A.S., 96, 1, 290-295, (1999)].

This family encompasses, besides *GAI*, the *RGA* [Silvestrone et al., Genetics, 146, 1087-1099, (1998)] and *SCARECROW* [Di Laurenzio et al., Cell, 86, 423-433, (1996)] genes of *Arabidopsis*, and also the tomato *LS* (lateral suppressor) gene [Shumacher et al., P.N.A.S., 96, 1, 290-295, (1999)]. At the current time, about twenty genes belonging to the GRAS family have been identified in *Arabidopsis*.

The proteins which make the GRAS family have a very variable N-terminal portion and a very conserved C-terminal portion with five recognizable motifs, in particular the VHIID motif.

The biological functions of most of these proteins are not yet precisely known, but their role as transcription factors is strongly presumed. The investigations carried out on the 4 most thoroughly studied genes at the current time (*SCR, GAI, RGA* and *LS*), show that these genes encode transcription factors involved in controlling the perception of and the response to gibberellins, and indicate the probable importance of this family in controlling the morphogenesis and the development of higher plants.

The inventors have now characterized and sequenced the *BZH* gene of *B. napus*, and its mutant allele *bzh*, associated with the dwarf phenotype previously observed by Foisset et al., (1995, abovementioned publication).

The sequence of the wild-type *BZH* gene is represented in the attached sequence listing under the number SEQ ID NO: 1, and the sequence of its translation product is represented under the number SEQ ID NO: 2. The sequence of the *bzh* mutant allele is represented in

the attached sequence listing under the number SEQ ID NO: 3, and the sequence of its translation product is represented under the number SEQ ID NO: 4.

The coding region of the *BZH* gene is 1716 bps and the corresponding protein is 572 amino acids.

Analysis of the sequences of the *BZH* gene and of its translation product make it possible to place it in the GRAS family, and in particular in the subgroup comprising GAI, RGA and RGA-like. The alignment of the polypeptide sequences deduced from the *BZH* genes, with other genes of the GRAS family, namely the *GAI, RGA, RGA-LIKE, SCARECROW* and *LS* genes, is represented in figure 1.

Analysis of the sequences of the *bzh* mutant allele and of its translation product shows that the *bzh* mutation is a G → A substitution at position 1695 of the coding sequence. It leads to a glutamic acid → lysine amino acid change at position 546 of the polypeptide sequence.

Surprisingly, the *bzh* mutation is totally different from the *gai* mutation of *Arabidopsis*. In particular, while the *gai* mutation of *Arabidopsis* affects a region located in the N-terminal portion of the GAI protein, the *bzh* mutation affects a region located in the C-terminal portion of the BZH protein.

A subject of the present invention is a nucleic acid sequence obtained by mutation of a sequence encoding a plant protein of the GRAS family comprising the following peptide sequence (I):

Gly Tyr X_1 Val Glu Glu (I)

in which X_1 represents arginine or asparagine, characterized in that said mutation results in a modification of said sequence (I).

The expression "modification of the sequence (I)" is in particular intended to mean the substitution of one or more amino acids of said sequence, the insertion of one or more amino acids into this sequence, or the deletion of all or part of said sequence.

Plant proteins of the GRAS family comprising the peptide sequence (I) are in particular the BZH proteins of rapeseed, and also the proteins of the GAI or RGA subfamilies described above.

According to a preferred embodiment of a nucleic acid sequence in accordance with the present invention, it encodes a mutant protein comprising the following peptide sequence (II):

$$\text{Gly Tyr } X_1 \text{ Val Glu } X_2 \qquad \text{(II)}$$

in which X_1 is as defined above, and X_2 represents an amino acid other than glutamic acid. Advantageously, X_2 represents a basic amino acid, preferably a lysine.

The invention in particular encompasses the nucleic acid sequences encoding the polypeptide represented in the attached sequence listing under the number SEQ ID NO: 4, for example the sequence of the *bzh* mutant allele which is represented in the attached sequence listing under the number SEQ ID NO: 3.

A subject of the invention is also plants with reduced development, comprising one or more copies of a nucleic acid sequence in accordance with the invention.

This encompasses in particular:

- mutant plants obtained from wild-type plants by conventional mutagenesis techniques, for example by treating seeds with a physical or chemical mutagen, selecting, from the plants derived from the treated seeds, the plants exhibiting dwarfism insensitive to gibberellins, and searching, among these plants, using conventional detection techniques of nucleic acid hybridization, for those which have a mutation in the nucleic acid sequence encoding the peptide sequence (I). It is also possible to introduce the desired mutation into a fragment, cloned beforehand, of the gene concerned and to reinsert the mutated sequence into the original gene as a replacement for the corresponding wild-type DNA;

- transgenic plants obtained by transgenesis of a host plant with a nucleic acid sequence in accordance with the invention;

- the descendants, possibly being obtained by sexual reproduction or vegetative multiplication, of the mutant plants or of the transgenic plants mentioned above.

Advantageously, plants in accordance with the invention are crucifers, and in particular Brassicacea, such as for example rapeseed, cabbage, turnip, brown mustard or Ethiopian mustard.

The plants expressing a nucleic acid sequence in accordance with the invention show, compared with the wild-type plants, a reasonably considerable reduction in size depending on the level of expression in said plant of the nucleic acid sequence in accordance with the invention. This level of expression in particular depends on the number of copies of the sequence. For example, in the case of rapeseed, the *BZH/bzh* heterozygous plants have an intermediate size between

that of the dwarf *bzh/bzh* homozygous plants and that of the wild-type *BZH/BZH* plants.

The plants according to the invention have, in particular in the case of rapeseed, the following advantages:

- the possibility of very early sowing, allowing the assimilation of nitrates, without the risk of stem elongation before winter;
- better resistance to the cold;
- better monitoring of the crop, due to a shorter size which facilitates plant-protection treatments;
- very good resistance to torrential rain;
- ease of harvesting.

The present invention will be more clearly understood with the aid of the further description which follows, which refers to nonlimiting examples describing the characterization of the rapeseed *BZH* gene and of a sequence in accordance with the invention derived from said gene.

EXAMPLE 1: CHARACTERIZATION AND SEQUENCING OF THE WILD-TYPE *BZH* GENE AND OF THE MUTANT *bzh* GENE:

The *BZH* gene was isolated on a 2352 base pair DNA fragment obtained from the "STELLAR" rapeseed line. This fragment contains a 1716 bps coding sequence, and the deduced polypeptide sequence is 572 amino acids. The coding sequence and the deduced polypeptide sequence are represented on the attached sequence listing under the numbers SEQ ID NO: 1 and 2, respectively.

In order to compare the sequence of the wild-type gene and of the *bzh* mutant allele, 5 lines were studied: wild-type PRIMOR (WTP), dwarf PRIMOR (DP), wild-type DARMOR (WTD), dwarf DARMOR (DD) and wild-type STELLAR (WTSTE).

The DNA fragments corresponding to the *BZH* locus were amplified on these lines, using primers derived from the sequence of SEQ ID NO: 1.

The comparison of the sequences of the amplification products obtained made it possible to establish that the only difference common to dwarf PRIMOR and dwarf DARMOR compared with the wild-type genotypes is a G → A substitution at position 1695 of the coding sequence. This substitution leads to a Glu→Lys amino acid change at position 546 of the peptide sequence.

The coding sequence carried by the nucleic acid fragment amplified from the dwarf primur line, and the corresponding peptide sequence, are represented in the attached sequence listing under the numbers SEQ ID NO: 3 and 4, respectively.

EXAMPLE 2: DETECTION OF THE *bzh* MUTANT ALLELE IN DWARF PLANTS

49 lines derived from the cross: dwarf DARMOR X YUDAL, and also the following pairs of [wild-type]/[bzh] isogenic lines: ISL1770/ISN1770, DOUBLOL/DOUBLOL-Bzh, GASPARD/GASPARD-Bzh and TAPIDOR/TAPIDOR-Bzh, were analyzed by PCR amplification of an approximately 400 bp region of the coding sequence, corresponding to the C-terminal portion of the protein, and polyacrylamide gel electrophoresis of the amplification products.

The lines with a "dwarf" phenotype exhibited, on the gel, a band characteristic of the presence of the G $\rightarrow$ A substitution.